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DEFECTIVE RECOMBINANT ADENOVIRUSES EXPRESSING

D1 CYTOKINES FOR ANTITUMOR TREATMENT

(INS D1)
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Cytokines are molecules (hormone s) produced by cells following an antigenic stimulation or an activation by other factors. The first cytokine which will has been produced is interleukin-1 (IL-1). It permits activation of the T cells which, in turn, start producing a whole battery of lymphokines, some of which are essential for the activation of the immune system and the defenses against viral or parasitic infections.

10 (INS D2) For some years, cytokines have been used in anticancer immunotherapy. Nevertheless, when they are administered systemically, a number of problems arise. IL-2, for example, produces quite substantial side effects. 15 it is rapidly metabolized, so that high doses have to be administered repeatedly.

Better administration routes which would increase their efficacy while decreasing their adverse effects are hence being sought.

20 (INS D3) D3 The subject of the invention is hence defective recombinant adenoviruses expressing one or more cytokines, as well as the use of these recombinant adenoviruses for making up pharmaceutical compositions, in particular antitumor compositions, more especially compositions which can be injected directly into solid tumors of the host.

The subject of th pr sent invention is def ctive recombinant adenoviruses, characteriz d in that they contain a defective, non-replicable adenovirus genome

into which one or more nucleic acid sequences coding for one or more cytokines, in particular lymphokines, are inserted under the control of one or more promoters capable of being recognised by the polymerases of human 5 cells, more especially of human tumor cells or of cells infiltrating these tumors.

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D4
047 The invention relates more especially to recombinant nucleic acids capable of being used for the production of such defective recombinant adenoviruses.

Such a recombinant nucleic acid is characterized in that it contains, on the one hand a genomic sequence of an adenovirus which is defective in that it lacks the sequences needed for its replication, but which nevertheless contains those sequences which, in this genome, are the carrier of the genetic information needed for the corresponding adenovirus to enter the cells which the latter is capable of infecting, as well as the set of essential sequences needed for encapsidation of this adenovirus, and on the other hand an insert containing a nucleic acid sequence coding for a cytokine, this insert being under the control of a promoter present in or previously inserted into the abovementioned genomic sequence.

Adenoviruses, in particular type 2 or 5 adenoviruses capable of infecting humans (or human adenoviruses), or alternatively serotype 4 and 7 adenoviruses, represent especially preferred vectors in the context of the present invention, on account, in particular, of the large size of the foreign DNA fragment which it is

possible to insert into the genome of these viruses.

Advantageously the abovementioned nucleic acid insertion sequence(s), coding for one or more predetermined cytokines, are contained in a defective adenovirus 5 genome lacking the essential nucleotide sequences needed for replication of these adenoviruses, and more especially the transactivators E1A and E1B and, where appropriate, the E3 region of the adenovirus, or alternatively its E1 and E3 regions.

In other words, the invention turns to good account the capacity of these defective recombinant adenoviruses to allow the insertion sequence they contain to be expressed in the cells they invade even when, on account of their defective character, they do not multiply therein. In other words, the objective of the invention is to cause cytokines to be secreted actually within the cells of the tumour to be treated (tumor cells themselves and cells, in particular lymphocytes, which infiltrate these tumors) when these cells have been 15 infected with these defective adenoviruses, especially when the latter are injected directly into the tumor. The cytokines produced will thus activate first and foremost, in situ, the cytotoxic cells infiltrating the tumor and those present in proximity to the tumor.

Regarding the sequence for insertion into the defective recombinant adenovirus genome, this may be chosen from all those which express a cytokine capable of exerting either a direct antitumor effect, or an activating effect on immunocompetent cells of the body, or both

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together.

Among these cytokines, the following may be mentioned as examples: IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, α interferon, γ interferon, tumor necrosis factor 5 alpha (TNF α) ([same term in French]).

The same recombinant adenoviruses may also be used in the case of certain diseases in which there is an immune deficiency and in the case of certain parasitic or viral diseases, especially γ , α interferon and/or GM-10 CSF), in particular by administration systemically or via cells, preferably human cells, taken in a state that allows them to be injected into humans, these cells having previously been infected with a recombinant defective adenovirus according to the invention.

15 The properties of some of these cytokines are recalled below.

Interleukin-1 (IL-1):

This is produced essentially by activated macrophages and monocytes. Its molecular weight is 20 approximately 17 kilodaltons. It displays several activities, including:

- a) a chemoattractive action on polymorphonuclear cells and macrophages (1, 2),
- b) an increase in the cytotoxic activity of 25 spontaneous cytotoxic (natural killer or Nk cells),
- c) an induction of fever following an infection,
- d) most especially, the activation of T cells for the production of other factors.

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Interleukin-2 (IL-2), Interleukin-4 (IL-4) and
Interleukin-5 (IL-5):

These are produced by activated T lymphocytes. The action of these cytokines is restricted to cells of the immune system, they cause their multiplication and their activation: IL-2 and IL-4 have been tested in antitumor immunotherapy in mice and in man. In mice, they act synergistically and cause tumor regression.

Interleukin-6 (IL-6)

This is produced by many cell types including T lymphocytes, macrophages, fibroblasts, etc. It induces the final differentiation of B lymphocytes, which become antibody producers.

Tumor Necrosis Factor α (TNF α) ([same term in French]):

This is a factor produced by macrophages. It has a dual action: a direct action on tumor cells causing their lysis and an activation of the immune system.

The use of TNF α in man must be carried out cautiously since a large number of cells possess the receptor for it: which accounts for the importance of inducing its secretion only locally, actually within the tumor, to limit its adverse effects on the other cells of the host.

Interleukin-3- (IL-3), Interleukin-7 (IL-7) and Colony
stimulating Factor (CSF).

These are hematopoietic growth factors. They are produced essentially by lymphocytes, monocytes and macrophages. They act at different levels of hematopoiesis, that is to say of the different stages of

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differentiation of marrow cells to blood cells. In addition, CSF exerts very substantial effects on the body's primary defences as regards the bacterial defences, luring the macrophages to the sites of infection and increasing their capacity for phagocytosis.

In combination with IL-2 and IL-4, GM-CSF proves to be an important antitumor factor.

γ Interferon (IFN- γ)

This is a factor produced by activated T cells; it is endowed with antiviral properties; it inhibits the multiplication of viruses and parasites and causes the lysis of infected cells and some tumor cells.

α Interferon (IFN- α)

Produced by T cells and monocytes, this displays an antiviral and lytic effect on infected cells. IFN- α has been used in immunotherapy against some types of cancer, including mesothelium [sic].

Naturally, the invention is not limited, as regards the choice of insertion sequences which can be used in adenoviruses according to the invention, to those sequences which have been identified above. Nevertheless, the latter are illustrative of the palette of possibilities which are afforded to the therapist, who is responsible for making the choice of the most suitable defective recombinant adenovirus to be used in the light of the nature of the tumours to be combated.

The invention also relates to pharmaceutical compositions comprising one or more recombinant vectors as described above, in combination with a

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pharmaceutically acceptabl vehicle, specially sterile, isotonic compositions which can be inj ct d directly into the tumors to be treated, or dry, in particular lyophilized, compositions which, by the addition of 5 sterilized water or of physiological saline as the case may be, enable solutions which can be injected directly into the tumors to be made up or reconstituted.

Direct injection of non-replicable, modified adenoviruses into the tumor affords the advantage, on the one hand of avoiding diffusion of the recombinant adenoviruses in the general circulation, with the consequent side effects liable to be exerted by the cytokines expressed in places other than on the sites where the manifestation of their action is sought, in this instance the tumor cells themselves or the cells, in particular lymphocytes, which infiltrate them or which are present in their immediate proximity. Preferably, the injection is carried out at the very least in at least one site of the primary tumor.

20 Neither is the invention limited to administra-
tion of the recombinant adenoviruses containing the
sequences coding for the cytokines of the kind in ques-
tion directly in the tumors. Any other administration
route permitting access of these recombinant adenoviruses
25 to the tumor to be treated may be envisaged. In particu-
lar, use may be made of cells which are compatible with
the host, for example human fibroblasts, preferably on s
previously removed from th host him- or herself.

The invention also relates to cell cultures, for

example cultured fibroblasts previously infected with recombinant nucleic acids, more especially the defective adenoviruses defined above. These infected cells, where appropriate attenuated or rendered immunologically inert, 5 for example by irradiation, contribute to the eradication of installed tumors when they are injected systemically. This injection may be envisaged either alone, or in addition to injection directly into the tumor.

The subject of the invention is also a method for 10 obtaining the recombinant adenoviruses described above, which comprises, after the actual step of construction of a vector by introduction of one or more insertion nucleic acid(s) into the genome of the initial defective adenovirus, a step of transformation of transformable 15 cell lines of higher eukaryotes (in particular of human or animal origin) themselves containing a separate nucleotide sequence capable of complementing the portion(s) lacking in the genome of the defective adenovirus and without which replication of the latter is prevented, 20 said separate sequence preferably being incorporated in the genome of the cells of said cell line.

As a preferred example of such cell lines, there may be mentioned line 293, a human embryonic kidney line which contains, integrated in its genome, the first 25 eleven percent of the left-hand end of the genome of a type 5 adenovirus (Ad5). This fraction can then complement defective recombinant viruses which carry deletions of this region. Such a production method is described, more especially, in European Patent Application

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After transformation of these cell lines, the defective adenoviruses thus multiplied and produced are recovered from the culture medium of the cells of these 5 lines and purified.

Further details of the present invention will be given in the description which follows of the possibilities of construction of a recombinant vector adenovirus containing at least one sequence coding for a 10 cytokine, especially a lymphokine.

I. METHODS

A) Cells and viruses

Ad-5-transformed human embryonic kidney cell line 293 (Graham et al., 1977) was used for the transfection 15 of DNA as well as for the multiplication and titration of adenovirus (Ad). In effect, cell line 293 complements the functions of the genes for E1A and E1B functions and permits the replication of defective Ad recombinants. For the construction of the recombinant Ad, human Ad5-d1324, 20 carrying deletions in the E1 region (3.9-10.5 m.u.) and E3 region (78.5-84.3 m.u.), was used (Shenk and Williams, 1984). Cell lines 293, Hela [sic] and Vero were maintained in an Eagle minimum essential culture medium with 10% of fetal calf serum.

25 b) Construction of plasmids permitting the expression of different cytokines

The eukaryotic expression vector pMLP10 has been described (Ballay et al., 1985). A derivative of this vector (pMLP18) was constructed by insertion of a

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sequence containing different single restriction sites downstream of the adenovirus major late promoter. These sites thus permit cloning of th different genes coding for the chosen cytokines under the control of the viral promoter. Downstream of this sequence containing these single restriction sites, the sequence containing th polyadenylation signal of the gene coding for the early antigens of SV40 virus were placed. The BgIII | - HindIII fragment of Ad5 is cloned downstream. This 3-Kbp sequence contains the gene coding for protein IX which is necessary for encapsidation of the viral genome exceeding 97% of its normal size, and permits subsequent in vivo recombination. Sequences coding for the genes for the different cytokines are isolated from plasmids obtained from different teams. These sequences, obtained after cleavage by means of different restriction enzymes, are introduced into the multiple cloning site of the expression vector described above (pMLP-18). The different plasmids designated pMLP-cytokine (IL-2, IL-4, and the like), which are used for obtaining the recombinant viruses as described in the following section, are thereby obtained.

c) Transfection of DNA and isolation of recombinant viruses

The Ad-cytokine defective recombinant adeno-viruses were obtained by in vivo recombination between the straight fragment of the viral genome previously cleaved with th r striction enzyme Cla I and the homologous sequence existing on the plasmids

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pMLP-cytokine described above. The mixture of the fragment of the viral genome (2.6 m.u. - 100 m.u.), purified after cleavage, and of the plasmid linearized with the restriction enzyme *Cla* I or *Pvu* I is transfected into 5 293 cells using the calcium phosphate precipitation method (Graham and Van der Eb, 1973). Cell plaques showing a cytopathic effect are isolated 10 days later and the virus was amplified in culture. The viral DNA was extracted by the Hirt procedure (Graham et al., 1977) and 10 the recombinant viruses were identified by mapping with restriction enzymes.

~~Figure 1 shows diagrammatically a construction of this type employing an insertion sequence coding for an interleukin (IL-2, IL-4, and the like).~~

15 In this figure:

- "leaders" corresponds to a tripartite leader
- "Del" corresponds to a "deletion"
- Ad dl 324 corresponds to an adenovirus provided with the abovementioned "deletions".

20 d) Expression of the sequences coding for an expressed cytokine

HeLa or Vero cell lines are infected with the defective recombinant viruses obtained. Cells effectively transfected may be characterized essentially by 25 means of detection of the activity of the cytokine released into their culture medium. In the case of IL-1, yields capable of reaching from 1 to 2 µg of interleukin per 10^6 cells have been observed.

Cells infected with an Ad-cytokine recombinant

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secrete variable amounts of the cytokine into the culture medium. Different methods exist for the detection and quantification of the cytokines produced.

1) Quantitative methods:

5 - ELISA, using specific antibodies
 - RIA (radioimmunoassay)
 - Western blotting

10 2) Qualitative methods (or biotests): based on
 the biological properties of cytokines

10 For example:

IL-2: Test of proliferation of CTL-L2 cells (CTL-L2 cells multiply and are maintained in culture only in the presence of IL-2 in the culture medium)

15 IL-3 and GM-CSF: Test of proliferation of TF-1 cells

IL-4: Test of proliferation of CTL-L2 cells and induction of soluble CD23 with certain cells including lymphocytes.

INF- α : Cytotoxicity test on L92-9 cells.

20 Neutralization test: The effect of cytokines may be blocked by incubating the target cells in the presence of cells of specific antibodies.

Some results obtained with the adenovirus vector carrying the IL-2 gene (Ad-IL-2) are described below.

25 1) Cells infected in vitro with Ad-IL-2 secrete significant amounts of functional IL-2.

2) Direct injection of the vector carrying the IL-2 gene into tumors already established in the animal (the tumor diameter at the time of injection is between 4 and

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7 mm) induces stimulation of the immune system which manifests itself in a stabilization of the size of the tumor, and then its regression to the point of complete disappearance in 40% to 50% of cases.

5 This result may be improved by treating the tumors in an earlier phase of its development or by using different vectors at the same time, for example combination of Ad-IL-2 with Ad-INF and/or Ad-IL-4, Ad-GM-CSF, Ad-IL-3. This combination has to be specified
10 according to the type of tumor.

3) Tumor cells infected in vitro and then injected into syngeneic animals or even immunodeficient animals (Nu/Nu mice) lose their tumorigenic power (at least up to 80% of the animals reject the tumor cells; in other words, the
15 tumor cells no longer proliferate in 80% of immunodeficient animals injected with these cells.

4) Animals which have rejected a first injection of infected tumor cells are highly immunized and are protected against parent (uninfected) tumor cells injected
20 at different times and at different places.

When coinjected with tumor cells infected in vitro, the spleen cells of these immunized animals are, furthermore, capable of transferring the antitumor immunity to recipient animals.

25 It is self-evident that the descriptions of constructions of recombinant defective adenoviruses envisaged above are in no way limiting in character. Other constructions may be produced, in particular

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according to the variants also mentioned below as examples.

1) Promoter exchange

The adenovirus major late promoter may be
5 replaced by other promoters which are ubiquitous but of
exogenous origin, such as:

- promoter contained in the LTR (long terminal repeat) of RSV (Rouse sarcoma virus)
- the promoter of the IE gene of CMV (cytomegalovirus)
- 10 - The MMTV (mouse mammary tumor virus) or metallothioneine inducible promoters.

Similarly, promoters permitting a more specific expression restricted to tumor cells, may be used, such as, for example:

15 15 - the promoter of the rep gene of parvovirus HI.

The invention also relates to a recombinant nucleic acid of the abovementioned type, characterized in that the genomic sequence of the adenovirus lacks its 5' end region downstream of the early promoter of the E1A 20 region of the adenovirus, and in that the sequence coding for the cytokine is placed under the control of this early promoter. This recombinant nucleic acid may also be employed in applications mentioned more especially in connection with recombinant DNAs in which the sequence 25 coding for the cytokine is placed under the control of the adenovirus major late promoter.

2) Simultaneous expression of several cytokine genes

3 types of constructions are described:

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- the cytokine genes are under the control of two promoters which ar either identical or different (MLP and RSV, for example) and are located following one another.
- 5 - the cytokine genes are under the control of separate promoters cloned into separate regions of the virus.